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Two Dimensional Multiwavelength Fluorescence Spectra of Dipicolinic Acid and Calcium Dipicolinate

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FOR THE DIRECTOR

//signed//
STEPHEN R. CHANNEL, DR-IV
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Dipicolinic acid (DPA) and the Ca2+ complex of DPA (CaDPA) are major chemical components of bacterial spores. With fluorescence being considered for the detection and identification of spores, it is important to understand the optical properties of the major components of the spores. In this paper we report in some detail on the room temperature fluorescence excitation and emission spectra of DPA and its calcium ion complex and comparison of the excitation-emission spectrum in a dry, wet paste and aqueous form. DPA solutions have very weak, if any, fluorescence and it is only slightly greater in the dry state. After the exposure to a broad source UV light of the DPA, wet or dry, we observe a large increase in fluorescence with a maximum intensity emission peak at around 440 nm for excitation light with wavelength around 360 nm. There is a slight blue shift in the absorption spectra of UV exposed DPA from the unexposed DPA solution. CaDPA in solution and dried show very slight fluorescence and a substantial increase of fluorescence was observed after UV exposure with emission peak around 410 nm for excitation around 305 nm. The detailed excitation-emission spectra presented here are necessary for better interpretation of the fluorescence spectra of bacterial spores where DPA is a major chemical component.

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INTRODUCTION

Dipicolinic acid (2,6-pyridinedicarboxylic acid or briefly DPA) and its salts are major constituents of bacterial endospores and are rather uncommon elsewhere in nature. In some cases, DPA constitutes between 5% and 17% of the dry weight of the spore[1,2]. The presence of DPA in bacterial spores was first reported by Powell [3] in 1953. It has been suggested that it is involved in spore dormancy[4,5], spore stability[6], germination[7], heat resistance[4,5,8-10] and ultraviolet (UV) and gamma irradiation resistance[11,12]. DPA is primarily located in the spore core and has been proposed to be tightly packed with the vital cell constituents, linked with proteins or amino acids, or intercalated with DNA. The powerful chelating properties of DPA and the presence of almost equi-molar quantities of calcium and DPA in endospores stimulated the hypothesis that Ca^{++} ion and DPA are for the most part combined in a molecular complex [13]. This hypothesis has recently been substantiated by comparison of the IR spectra of DPA+ vs DPA- spores of *Bacillus subtilus* [14]. Bacterial spores all absorb in the region of DPA and CaDPA absorption [15,16], and the Raman spectra of spores show strong evidence of CaDPA [17].

Previous fluorescence excitation-emission studies by Nudelman et al. and Bronk et al. for DPA, CaDPA and DPA- ion in aqueous solution[18-21] indicated the minimal or no fluorescence for DPA prior to UV irradiation but substantial fluorescence after irradiation at 255 nm. The present study is motivated by the fact that more detailed excitation-emission studies are needed to understand the fluorescence of endospores. This is particularly true for dry or partially dry DPA salts because other studies in this lab indicate that there are substantial qualitative changes in the excitation emission spectra as spores are drying. We note that the DPA and CaDPA in the spore correspond more nearly to a solid sample or a wet paste than to DPA in solution. The previous studies did not include fluorescence from DPA in this form. Thus, further experimental measurements of multi wavelength fluorescence spectra with wet paste, dry crystalline and solution preparations of DPA and CaDPA are presented in this study.

MATERIALS AND METHODS

DPA in powder form was purchased from Sigma-Aldrich corp (St. Louis, Missouri, USA) and used as such. $CaCO_3$, $Ca(NO_3)_2$ and $Ca(OH)_2$ were obtained from Aldrich chemical company (Milwaukee, WI, USA). DPA solutions were prepared freshly each day with deionized water. Calcium dipicolinate (CaDPA) was prepared by mixing solutions of DPA with various calcium compounds. We measured the UV absorption spectrum of each sample and confirm the formation of CaDPA by its characteristic absorption spectrum[16]. Absorption measurements were made at room temperature with a Cintra 40 spectrophotometer (GBC, Dandenong, Vic, Australia) with matched 1 cm path length quartz cuvettes.

Initially, the fluorescence emission spectrum of a saturated DPA solution at room temperature was measured. We confirmed that this solution had no measurable fluorescence. Then the solution in the 1 cm quartz cuvette was exposed to broad-band UV light for 15 minutes. The full UV and visible spectrum of light from an 450 W ozone free xenon arc lamp (the fluorometer lamp) was used. We measured the UV fluence between 200 and 400 nm to be approximately 100 J/sq. cm with the 15 minute exposure. Immediately after exposure, the fluorescence spectra of the exposed solutions were measured. The exposed solution was then allowed to stand for 3-4 hours in the dark before another measurement was made.

Additional sets of DPA solutions with different calcium compounds were prepared and the absorption spectra and the fluorescence spectra of each sample of calcium dipicolinate were measured. Then each of these solutions was exposed to the UV arc lamp for 15 minutes and the absorption and fluorescence spectra were measured. The variation of fluorescence intensity of the exposed solution as a function of time was also measured.

Pieces of filter paper (GF/A, Glass Microfibre Filters, Whatman, Kent, UK) were cut about 1 cm wide and 3 cm high. The solutions of DPA and CaDPA were pipetted onto the filter paper. This particular filter paper was selected after making measurements of the fluorescence from many different types of filter paper. The chosen filter paper has a minimal fluorescence signal that does not overlap significantly with the DPA or CaDPA fluorescence. By multiple cycles of pipetting the solution onto the filter paper and allowing the filter paper to dry, wet and dry samples of solid or wet paste DPA and CaDPA sufficiently concentrated for fluorescence measurements were made. The filter paper with the wet or dried sample was placed at 45 degrees to the incident and fluorescence light in a 1 cm path length quartz cuvet and placed in the spectrofluorometer.

The spectrofluorometer (SLM 8000C, Urbana, IL USA) was configured with single monochromators with an Eagle design with stigmatic concave diffraction grating 900 L/mm, Blazed at 300 nm for excitation and 400 nm for emission. The slits on both monochromators were set for a 4 nm bandwidth. We use a scan rate of 600 nm per minute. The excitation wavelength used to create each emission spectrum was varied in 10 nm increments from 200 to 600 nm. The emission spectrum was stepped every 1.0 nm from 10 nm longer than the excitation wavelength to 10 nm shorter than twice the excitation wavelength. All emission spectra presented in this paper were acquired with identical instrument parameters.

The primary analysis of the spectra was done with *Mathematica* (Wolfram research, Urbana, IL). Two dimensional excitation/emission plots were created by dividing the wavelengths of the emission spectrum by the excitation wavelength for each spectrum. This creates a common unit less range of approximately 1.0 to 2.0 for each spectrum. Mathematica then interpolates between the emission spectra in calculating the contour plot.

DPA

In Fig. 1A we show the 2-D multiwavelength fluorescence spectra of a DPA solution before exposure to the UV arc lamp. We did not observe any fluorescence, but did observe the Raman scattering peak from water. The fluorescence of the same solution exposed to approximately 100 J/sq.cm of UV light is shown in Fig. 1B. The DPA solution was exposed to UV light by an ozone free xenon arc lamp for 15 minutes and allowed to stand 3-4 hours in the dark before the fluorescence measurements were made. The noise in Fig. 1A is 0 to 3 "counts" (arbitrary units of intensity). The peak in Fig. 1B has a maximum of 90 counts. The peak has been enhanced about a factor of approximately 45 by the UV light. The fluorescence observed in Fig. 1B has a peak emission wavelength at 440 nm and occurs with an excitation wavelength of 360 nm. The DPA solution did show a well-defined absorption peak around 277 nm as can be seen in Fig. 2.

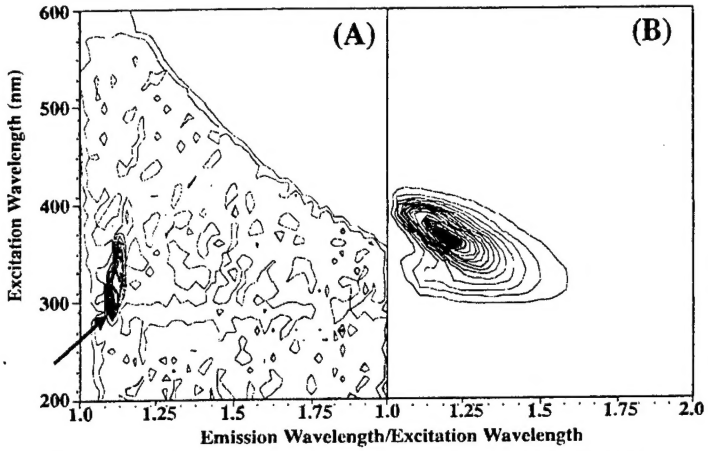

Figure 1: **(A)** Luminescence spectrum of a freshly prepared DPA solution. The arrow points to the Raman scattering from the water. **(B)** Fluorescence spectrum of the same DPA solution after UV exposure. The excitation was stepped every 10 nm from 200 to 600 nm. The intensity is equally divided by 15 contour lines with the peak intensity for each given sample controlling the scale. The greatest intensity is dark and the least intensity is white.

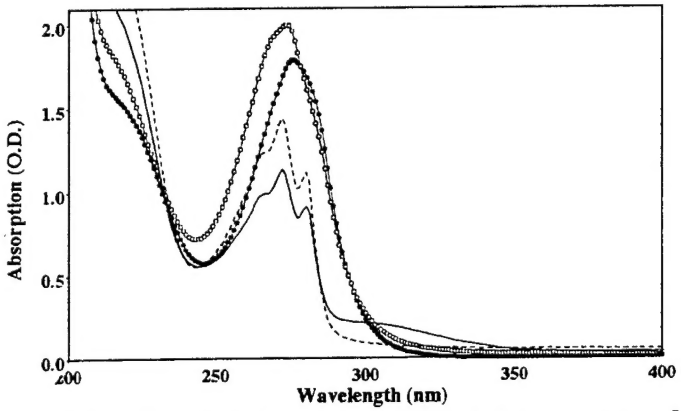

Figure 2: The absorption spectra of DPA before exposure to UV light (filled in circles) and DPA after exposed to about 100 J/sq. cm of UV light (open circles). Also the absorption of calcium DPA before exposure to UV light (dashed line) and after exposure to about 100 J/sq. cm of UV light (solid line). All samples were solutions measured in a 1 cm quartz cuvet. The same sample was measured before and after exposure.

We note that the absorption spectrum of DPA shifts slightly to shorter wavelengths upon exposure to UV light (see Fig. 2). There is also a very small increase in the absorption in the 300 to 350 nm region. This very small increase does not help to explain the strong fluorescence maximum observed at 360 nm. At this point, we are unable to explain the enhancement in the fluorescence. The changes in the absorption spectra and the well-defined fluorescence emission as a result of UV exposure and the retention of the fluorescence intensity after a week in the dark indicate an irreversible photochemical transformation.

Since the DPA in spores is likely to be similar to the dry crystalline state or a wet paste, we measured the fluorescence from the exposed DPA dried on filter paper to make a wet paste or a dry sample. Drying was done at room temperature and in the dark. The fluorescence for the wet paste is shown in Fig. 3A. The fluorescence from dry crystals of exposed DPA is shown at Fig. 3B. Both of these spectra are similar to Fig. 1B (the UV exposed solution of DPA), with the fluorescence spread over a slightly wider spectral range.

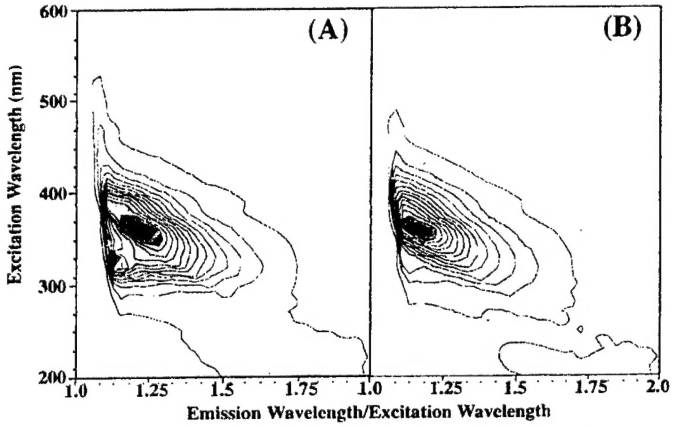

Figure 3: **(A)** Fluorescence spectrum of UV exposed DPA as a wet paste on filter paper. **(B)** Fluorescence spectrum of UV exposed DPA as dry crystals on filter paper. The sample preparations are described in the text. The excitation was stepped every 10 nm from 200 to 600 nm. The intensity is equally divided by 15 contour lines. The greatest intensity is dark and the least intensity is white.

We then took crystals of DPA, not exposed to UV light and placed them on the filter paper and measured the fluorescence directly. This is shown in Fig. 4A. Probably due to the much greater amount of dry DPA exposed on the filter paper compared to the experiments in solution, a fluorescence spectrum can be measured, even without UV exposure. The small trail of peaks that occur for short wavelength excitation is an anomalous signal at 420 nm that is observed whenever a large amount of light is reflected into the detection monochromator of the fluorometer. In this sample, the white filter paper and the DPA crystals both scatter a large amount of light into the detection monochromator.

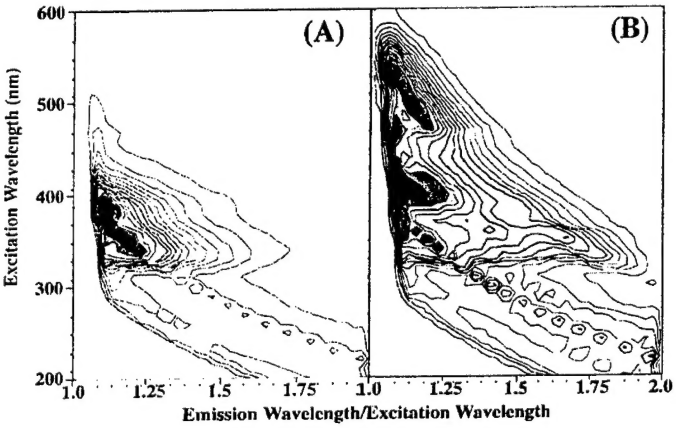

Figure 4: **(A)** Fluorescence spectrum of dry DPA without UV exposure on filter paper. **(B)** Fluorescence spectrum of UV exposed dry DPA crystals on filter paper. The excitation was stepped every 10 nm from 200 to 600 nm. The intensity is equally divided by 15 contour lines. The greatest intensity is dark and the least intensity is white. The maximum intensity on **(B)** is twice as large as the maximum intensity on **(A)**.

One might question if the fluorescence of the dry crystals of DPA is enhanced compared to the small-to-zero fluorescence observed from a DPA solution. So, we carefully wet the filter paper with deionized water while it was in the cuvet and remeasured the fluorescence. The fluorescence decreased nearly a factor of three after the DPA was wet. Since some of the decrease could have been due to DPA going into solution and being washed away from the portion of the filter paper probed by the fluorometer, we dried the same piece of filter paper with the DPA in the dark and the signal increased by a factor of two. From this, we conclude

that there is a measurable increase of fluorescence for DPA when it dries. Dry DPA exhibits about twice the fluorescence of wet DPA.

Finally, we exposed the dry DPA crystals on the filter paper to the 100 J/sq. cm of broadband UV light. The UV enhanced fluorescence spectrum is shown in Fig. 4B. The UV enhancement of the peak fluorescence was a factor of 1.8. The integrated fluorescence over all wavelengths increases by a factor of ~3.8. The UV exposed DPA crystals show a broad fluorescence spectrum and very long wavelength fluorescence that was not observed for unirradiated DPA.

CaDPA

In Fig. 5A we show a modest fluorescence signal for DPA solution mixed with $CaCO_3$ with excitation in the range of 300 nm and emission peaking at about 400 nm. The arrow in the figure points to the Raman scattering of the water. During the sample preparations the solution is exposed to room light. Also, during the fluorescence measurements, which takes about 90 minutes, the solution is exposed to UV light from the fluorometer. Multiple fluorescence scans of the same CaDPA solution shows a slight increased fluorescence, indicated that the UV light from the fluorometer is affecting the solution and enhancing the fluorescence. (We always verified presence of CaDPA by comparing absorbance with the compounds characteristic absorbance—see Fig. 2)

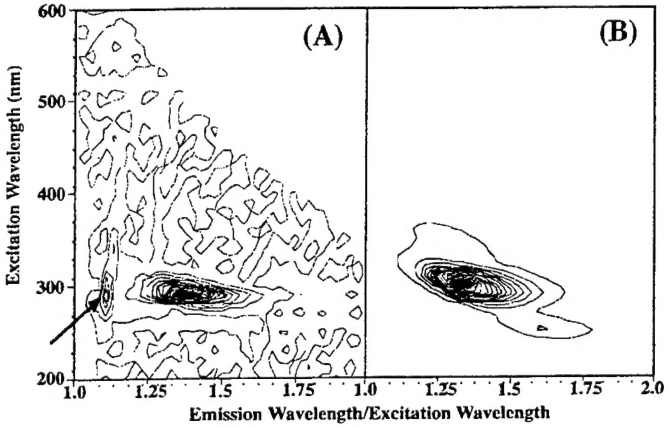

Figure 5: **(A)** Luminescence spectrum of a freshly prepared CaDPA solution. The arrow points to the Raman scattering from the water. **(B)** Fluorescence spectrum of the same CaDPA solution after UV exposure. The excitation was stepped every 10 nm from 200 to 600 nm. The intensity is equally divided by 15 contour lines. The greatest intensity is dark and the least intensity is white.

We did not buffer the solution to hold the pH constant. This was to prevent the presence of buffer salts from interfering with measurements of dried and partially dry DPA salts. Previous spectrophotometric methods [16] for CaDPA estimation used high pH's to ensure the stability of the chelate. These high pH's favour precipitation of calcium carbonates and phosphates, causing turbidity and a loss of DPA by co precipitation. A more recent method [22] using 100 mM Tris buffer at pH 7.6 provides adequate buffering for the acid extract, and a Ca^{2+} concentration required to achieve essentially complete formation of CaDPA chelate. Other researchers have reported that the reproducibility and consistency of the fluorescence intensity were found to be highly pH dependent [18].

The CaDPA solution was exposed to UV light (~ 100 J/sq. cm using the 15 min. exposure and 2 hour resting period) and measured the fluorescence. We observed a large increase of fluorescence in the same range of emission and excitation as CaDPA without exposure (see Fig. 5B). The peak fluorescence increased by a factor of 85 after exposure. The total volume under the curves increased by a factor of 50. As shown in Fig 2 the exposed absorption spectrum of CaDPA shows only minor changes compared with the absorption spectrum of an unexposed CaDPA solution. Nudelman et al, [18] reported that low-dose treatment with 254 nm UV irradiation caused only slight changes in the absorption spectrum of CaDPA. However, irradiation of 10 uM solutions of CaDPA in HEPES buffer at higher intensity and for longer durations, resulted in absorption spectra that changed substantially. The CaDPA absorption spectrum also varies with temperature [16]. The changes that we observe in the CaDPA fluorescence remain constant for at least seven days after exposure. The fluorescence measurements were reproducible qualitatively and quantitatively even without using the buffer.

Similar fluorescence and absorption spectra of CaDPA were obtained when solutions were made of DPA with different calcium compounds ($Ca((NO)_3)_2$, $Ca(OH)_2$) (data not shown). All samples were prepared on the day of the experiment. We also measure the fluorescence of CaDPA crystals in dry and wet form. We could not see any major difference in the fluorescence spectra of CaDPA in aqueous, dry and wet form other than a small increase in the spectral width of the fluorescence peaks (Figs. 6A and B). Similar to the DPA, we measured about a factor of 3.7 increases in the fluorescence of the dry crystals of CaDPA compared to wet crystals (data not shown).

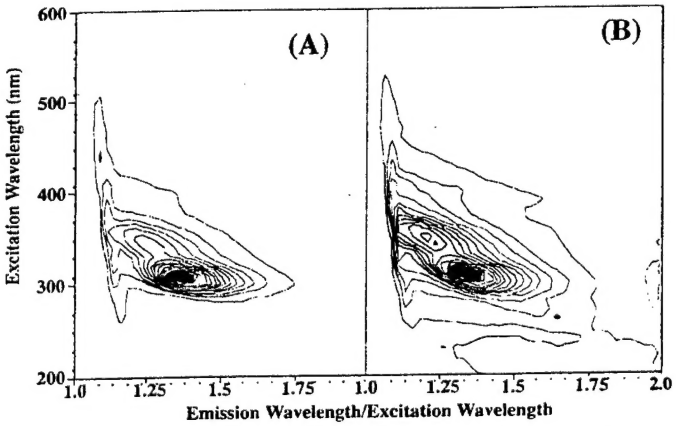

Figure 6: **(A)** Fluorescence spectrum of UV exposed CaDPA as a wet paste on filter paper. **(B)** Fluorescence spectrum of UV exposed CaDPA as dry crystals on filter paper. The sample preparations are described in the text. The excitation was stepped every 10 nm from 200 to 600 nm. The intensity is equally divided by 15 contour lines. The greatest intensity is dark and the least intensity is white.

We observe a peak similar to the peak measured from exposed DPA in both the dry and wet form of CaDPA spectrum. Some of the DPA in the mixture might not have formed a complex with calcium due to an excess amount of DPA or an insufficient excess of calcium ions. Okay.

When dry CaDPA was given our standard UV exposure it also showed enhanced fluorescence. As with the dry DPA samples, enhancement was much less for the dry sample compared to the solution. Unlike the dry DPA, no major changes (other than the intensity change) in the fluorescence spectrum was observed for the UV exposed dry CaDPA sample.

The dry CaDPA before and after UV irradiation is shown in Figs. 7A and B. The peak fluorescence increased by a factor 1.5.

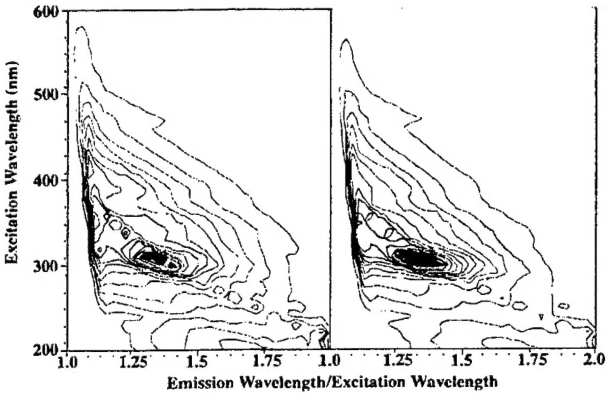

Figure 7: **(A)** Fluorescence spectrum of dry CaDPA without UV exposure on filter paper. **(B)** Fluorescence spectrum of UV exposed dry CaDPA crystals on filter paper. The excitation was stepped every 10 nm from 200 to 600 nm. The intensity is equally divided by 15 contour lines. The greatest intensity is dark and the least intensity is white. The maximum intensity on **(B)** is 1.5 times larger than the maximum intensity on **(A)**.

CONCLUSION

Using autofluorescence spectra, we extended observation of the spectral properties of DPA and CaDPA in aqueous solution to similar observations with dry crystals and wet paste at room temperature. The results of the spectral properties of these chemicals are necessary for the interpretation of the two dimensional fluorescence studies of bacterial spores. Upon UV exposure the fluorescence of DPA and CaDPA increases dramatically for dry as well as wet forms. We could not see any substantial qualitative changes in the DPA exposed as dry and wet crystals although drying the DPA increased the observed fluorescence and the cystals demonstrated a slightly broader emission peak. We observe that multiple-wavelength excitation fluorescence provides an additional means for distinguishing DPA and CaDPA.

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